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Abstract

MicroRNAs (miRNAs) are endogenously expressed small noncoding RNAs that act as posttranscriptional regulators of gene expression. Dysregulation of these molecules has been observed in many types of cancers. Altered expression levels of several miRNAs were identified also in gliomas. It was been frequently shown that miRNAs are involved in core signaling pathways, which play key roles in cellular processes, such as proliferation, apoptosis, cell cycle regulation, invasion, angiogenesis, and stem cell behavior. Therefore, miRNAs have a great potential to act as new class of diagnostic, prognostic, and predictive biomarkers as well as promising therapeutic targets in gliomas. Here, we summarize the current knowledge about miRNAs significance in glioma molecular pathology, with special focus on their involvement in core signaling pathways, their roles in drug resistance, and their potential clinical implications.

Keywords

MicroRNA • Transcription • Signal transduction

Abbreviations

BMI1	BMI1 polycomb ring finger oncogene
Cdk	Cyclin-dependent kinase
c-MYC	v-myc avian myelocytomatosis viral oncogene homolog
EGFR	Epidermal growth factor receptor
GBM	<i>Glioblastoma multiforme</i>

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GSC	Glioma stem cell		
IFN	Interferon		
IKK $\alpha/\beta/\gamma$	Conserved helix-loop-helix ubiquitous kinase $\alpha/\beta/\gamma$		
IRS	Insulin receptor substrate		
JAK-STAT	Janus kinase-signal transducer and activator of transcription		
miRNA	MicroRNA		
MMP	Matrix metalloproteinase		
NF- κ B	Nuclear factor of kappa light polypeptide gene enhancer in B-cells		
p21CIP1	Cyclin-dependent kinase inhibitor 1A		
p27	p27 protein		
PDGFR	Platelet-derived growth factor receptor		
PI3K/AKT	Phosphatidylinositol-4,5-bisphosphate thymoma viral oncogene homolog)	3-kinase/v-akt	murine
PLD2	Phospholipase D2		
PTEN	Phosphatase and tensin homolog		
RAS	Rat sarcoma viral oncogene homolog		
TMZ	Temozolomide		
VEGF	Vascular endothelial cell growth factor		
VEGFR	VEGF receptor		
WHO	World Health Organization		

4.1 Introduction

Glial tumors of adults are the most frequently occurring primary tumors of central nervous system with a tendency to invade the surrounding brain tissue, originating in astrocytic glial cells. They are traditionally divided into histopathological subtypes defined by World Health Organization (WHO) classification. Numerous investigations have indicated that the improved understanding of the biology and molecular factors involved in the development, progression, and drug resistance of gliomas will be necessary for prediction of clinical outcome and therapy response, and mainly for development of new therapeutic strategies. Only a few molecular biomarkers have been validated and widely accepted in clinical practice till now and despite introduction of novel therapeutic approaches (bevacizumab, cilengitide) this cancer remains associated with very poor prognosis.

MicroRNAs (miRNAs) are endogenously expressed small noncoding RNAs that act as posttranscriptional regulators of gene expression. Deregulation of miRNAs has been observed in various types of cancers, including gliomas. There is continuously increasing evidence showing miRNAs involvement in core signaling pathways of glioma pathogenesis and their great potential as biomarkers and therapeutic targets.

4.2 MicroRNAs Function and Biogenesis

MiRNAs are a class of small 18–25 nucleotides long noncoding RNA molecules, whose function is to regulate gene expression on the posttranscriptional level. This regulation is mediated by the binding of miRNA to the 3'-untranslated region (3'UTR) of its target mRNA that induces either direct mRNA chain degradation or translational inhibition followed by deadenylation and mRNA decay. The decision on the fate of the target molecule is dependent on the degree of complementarity of both molecules. Perfect base pairing leads to the degradation in RISC, whereas imperfect bond leads first to the inhibition of mRNA translation subsequently followed by the degradation of target molecule due to its instability. Being able to regulate the translation even in case of imperfect complementarity causes that a single miRNA can regulate multiple mRNAs while a single mRNA can be targeted by different miRNAs. To date, over 1,400 human miRNAs have been identified (Griffiths-Jones et al. 2008). MiRNA expression is largely tissue and cell type specific and, moreover, these molecules play important role in a wide range of biological processes, such as development, differentiation, proliferation, and apoptosis (Niyazi et al. 2011). It is not surprising that deregulation of miRNAs was described as an important step in initiation as well as in progression and metastasis of many cancers including gliomas. On the basis of the target molecules, miRNAs can be classified as onco-miRs or tumor-suppressive miRNAs; some miRNAs may exhibit both features dependently on cellular context in various cancers (Chen 2005).

Most miRNA genes have their own promoters and are transcribed as autonomous transcription units (Carthew and Sontheimer 2009). Primary transcripts (pri-miRNAs) are generally transcribed by RNA polymerase II. Pri-miRNA can be up to 10 kb long and is characterized by the formation of stem loop structures. Single pri-miRNA frequently contains polycistronic cluster of miRNAs that are co-transcribed (Lawler and Chiocca 2009). In the nucleus, the pri-miRNA precursor is further cleaved through the activity of an enzyme called Drosha and cofactor DGCR8/Pasha. This processing results in a precursor miRNA (pre-miRNA) that is usually between 60 and 100 nucleotides in length. The pre-miRNA is exported from the nucleus in a RanGTP-dependent process after binding to transporter Exportin-5. In the cytoplasm, pre-miRNA is processed by the enzyme Dicer and, subsequently, one (passenger) of two chains of mature duplex miRNA is destroyed while guide chain is stabilized by the Argonaute proteins and incorporated into RISC (RNA-induced silencing complex). Recent findings show that guide chain can be derived from both the 5' and 3' ends of pre-miRNA hairpin depending on the thermodynamic stability on the 5' end of the mature miRNA (Schwarz et al. 2003; Ladomery et al. 2011). The RISC complex with a miRNA guide strand is known as a miRISC complex. MiRNA involved in this macromolecular complex is prepared to perform its regulatory effects.

4.3 MicroRNAs in Glioma Pathogenesis

Tumor behavior is largely dependent on the abilities of the individual tumor cells to have the capacity of uncontrolled proliferation, to regulate own cell cycle, to escape an apoptosis, to regulate angiogenesis, and to migrate and invade in the surrounding tissues. The other specific properties such as the capability to self-renewal, unlimited proliferation potential, and differentiation have been observed in glioma stem cells. It has been described many times that miRNAs are involved in the regulation of these processes in many cancers including gliomas and, thus, these molecules can significantly influence glioma pathogenesis.

4.3.1 MicroRNAs in Proliferation, Migration, and Invasiveness

Among the key signaling pathways regulating cell proliferation, migration, and invasiveness belong EGFR (epidermal growth factor receptor), PI3K/AKT (phosphatidylinositol-4,5-bisphosphate 3-kinase/v-akt murine thymoma viral oncogene homolog), and NF- κ B (nuclear factor of kappa light polypeptide gene enhancer in B-cells) (Nikaki et al. 2012). These signalizations have been many times described as deregulated in gliomas and, thus, deeper understanding of them could help discover new therapeutic approaches in initial as well as recurrent diseases of glial origin. The EGFR signaling network contributes to promotion, progression, and metastasis of the wide range of solid cancers, including gliomas. It is not surprising that this pathway is regulated by many miRNAs, which are therefore promising therapeutic targets (Fig. 4.1).

Mechanistic studies identified gene targets of miR-21 among important components of the EGFR signaling pathway. GBM (*glioblastoma multiforme*) cell lines U251 and LN229 characterized by mutated and wild-type PTEN (phosphatase and tensin homolog), respectively, showed a decreased expression of EGFR, activated AKT, Cyclin D, and Bcl-2 (B-cell CLL/lymphoma 2) after silencing of miR-21 expression (Zhou et al. 2010a; Sana et al. 2011). Although miR-21 is known to regulate PTEN and downregulation of miR-21 led to increased PTEN expression, the GBM suppressor effect of antisense miR-21 is most likely independent of the PTEN status because U251 has mutated PTEN (Ren et al. 2010b; Zhou et al. 2010a; Sana et al. 2011). In addition to the PTEN, miR-21 regulates proliferation, migration, invasiveness, and other cell processes through direct or indirect repression of many other cancer genes (Gabriely et al. 2008; Gaur et al. 2011a). Interestingly, significant synergistic inhibition of proliferation and invasiveness was observed after co-inhibition of miR-21 and miR-10b. It was hypothesized that miR-21 inhibitor may interrupt the activity of EGFR pathways, increasing PDCD4 [programmed cell death 4 (neoplastic transformation inhibitor)] and TPM1 [tropomyosin 1 (alpha)] expression and reducing MMP (matrix metalloproteinase) activities, independently of PTEN status. Meanwhile, miR-10b inhibitor induces translational suppression of the mRNA encoding HOXD10 (homeobox D10) leading to the increase of the expression of the well-

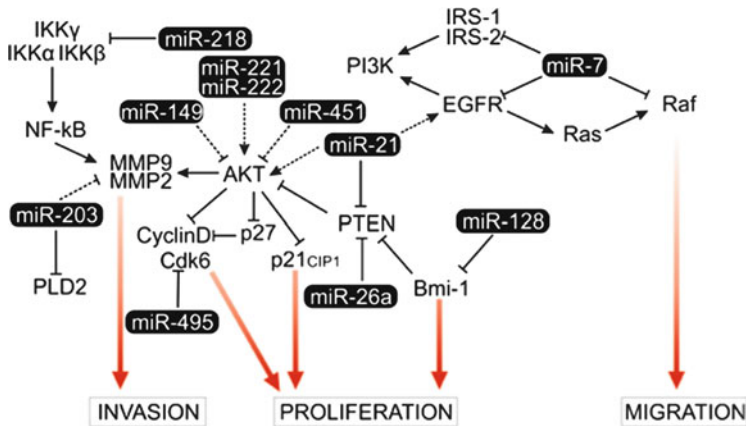


Fig. 4.1 MicroRNAs involved in regulation of glioma proliferation, migration, and invasion. *Bmi-1* BMI1 polycomb ring finger oncogene; *Cdk6* cyclin-dependent kinase 6; *EGFR* epidermal growth factor receptor; *IKK $\alpha/\beta/\gamma$* conserved helix-loop-helix ubiquitous kinase $\alpha/\beta/\gamma$; *IRS-1/2* insulin receptor substrate 1/2; *MMP2/9* matrix metalloproteinase 2/9; *NF- κ B* nuclear factor of kappa light polypeptide gene enhancer in B-cells; *p21/CIP1* cyclin-dependent kinase inhibitor 1A; *p27* p27 protein; *PI3K* phosphatidylinositol-4,5-bisphosphate 3-kinase; *PLD2* phospholipase D2; *PTEN* phosphatase and tensin homolog; *Raf* zinc fingers and homeoboxes 2; *Ras* rat sarcoma viral oncogene homolog. —| direct suppression; —> direct activation; - -| indirect suppression; - -> indirect activation

characterized pro-metastatic gene RHOC (ras homolog family member C). Taken together, combination of miR-21 inhibitor and miR-10b inhibitor could be an effective GBM therapeutic strategy (Dong et al. 2012).

PTEN downregulation followed by AKT activation was described after transfection of GBM cells with the miR-26a-2 primary transcript. Similarly, the miR-26a mimics decreased PTEN protein levels and increased AKT phosphorylation (Huse et al. 2009; Kim et al. 2010; Sana et al. 2011). Another research group also showed that ectopic expression of miR-26a influenced cell proliferation by targeting PTEN and suggested that miR-26a is regulated by transcription factor c-MYC (v-myc avian myelocytomatosis viral oncogene homolog) (Guo et al. 2013).

Modulation of AKT signaling cascade using miRNAs in GBM cell lines was described also by Nan et al. In this study, transfection of miR-451 mimic reduced expression levels of many molecules including AKT1, MMP-2, and MMP-9. According to phenotypic experiments, miR-451 inhibited invasive ability and cell proliferation in GBM cells in vitro (Nan et al. 2010; Sana et al. 2011). Another miRNA involved in the EGFR signaling pathway is miR-7. Kefas et al. showed that miR-7 directly inhibits EGFR expression and independently suppressed the AKT pathway via targeting upstream regulators IRS-1 (insulin receptor substrate) and IRS-2. Moreover, transfection with miR-7 oligonucleotides decreased viability and invasiveness of primary GBM cell lines (Kefas et al. 2008; Webster et al. 2009). Webster et al. also described Raf1, another member of the EGFR signaling pathway, as a direct target of miR-7 in cancer cells (Webster et al. 2009).

Godlewski et al. published that miR-128 expression significantly reduced glioma cell proliferation in vitro and glioma growth in vivo. This effect was explained by direct regulation of the BMI1 (BMI1 polycomb ring finger oncogene), which is significantly upregulated in GBM compared to normal brain while miR-128 showed an opposite regulation. Moreover, miR-128 expression caused a decrease in histone methylation and AKT phosphorylation, and upregulation of p21(CIP1) (cyclin-dependent kinase inhibitor 1A) levels, consistent with BMI1 downregulation (Godlewski et al. 2008).

Finally, miR-221 and miR-222 were revealed as potential regulators of many target genes involved in AKT signaling pathway. Upregulation of miR-221/222 resulted in remarkable increase of p-AKT (phosphorylated AKT) and significant changes in the expression of AKT-related genes in glioma cells. Consequently, miR-221/222 overexpression increased glioma cell proliferation and invasion in vitro and induced glioma growth in mouse model. These results suggest that miR-221/222 enhance glioma malignant phenotype via activation of the AKT signaling pathway (Zhang et al. 2010b; Sana et al. 2011). Another study revealed that phenotypic effect of miR-221/222 is caused at least in part by targeting Cx43 (gap junction protein, alpha 1, 43 kDa), which has been identified as a tumor suppressor and major component for the establishment of GJIC (gap junction intercellular communication) in glial cells (Hao et al. 2012).

Another signaling pathway mentioned at the beginning of this chapter is the NF- κ B pathway. NF- κ B is the transcription factor with pleiotropic activity owing to its central roles in various biological processes. Aberrant activation of NF- κ B signaling pathway has been proved to be important for invasiveness and metastatic capacity of tumors through upregulation of MMPs and transcription factors regulating E-cadherin, such as Snail (snail family zinc finger 1), Twist (twist family bHLH transcription factor), or Slug (snail family zinc finger 2). A critical component in NF- κ B regulation is the IKK- β (conserved helix-loop-helix ubiquitous kinase) complex (Ghosh and Karin 2002; Song et al. 2010). Song et al. identified miR-218 expression in glioma cell lines and in human primary glioma tissues was substantially downregulated, when compared to miR-218 expression in normal human astrocytes and normal brain tissues. Forced upregulation of miR-218 dramatically reduced the migratory speed and invasive ability of analyzed cells. Ectopic expression of miR-218 downregulated MMP-9 and reduced NF- κ B transactivity at transcriptional level, whereas inhibition of miR-218 has the opposite effect. Authors also demonstrated that miR-218 could inactivate NF- κ B/MMP-9 signaling by directly targeting IKK- β (Song et al. 2010; Sana et al. 2011).

High-throughput analysis of the effects of 319 miRNA precursor molecules on cell proliferation in three (A172, LN405, and U87MG) GBM cell lines revealed nine miRNAs (miR-129, miR-136, miR-145, miR-155, miR-181b, miR-342-5p, miR-342-3p, miR-376a, and miR-376b), which had an antiproliferative effect in glioma cells. Moreover, six miRNA target genes [*ROCK1* (Rho-associated, coiled-coil containing protein kinase 1), *RHOA* (ras homolog family member A), *MET* (met proto-oncogene), *CSF1R* (colony-stimulating factor 1 receptor), *EIF2AK1* (eukaryotic translation initiation factor 2-alpha kinase 1), and *FGF7* (fibroblast

growth factor 7)] were subsequently validated for the similar effects (Haapa-Paananen et al. 2013).

MiR-124 is another miRNA that plays an important role in regulating proliferation and invasiveness of GBM cells. As one of the possible mechanisms of these effects was revealed signalization through PPP1R3L, an inhibitory member of the apoptosis-stimulating protein of p53 family (IASPP), which is also able to affect growth, cell cycle progression, apoptosis, and metastasis of various types of cancer (Zhao et al. 2013b). Similarly, miR-203 inhibits the proliferation and invasion of U251 GBM cells partially via direct targeting of PLD2 (phospholipase D2) and/or ROBO1 [roundabout, axon guidance receptor, homolog 1 (*Drosophila*)]/ERK (mitogen-activated protein kinase 1)/MMP-9 signaling pathway (Dontula et al. 2013; Chen et al. 2013d). The ability of miR-100 to reduce proliferation *in vitro* was subsequently demonstrated *in vivo* where this miRNA treatment improved overall survival of treated animals. As a direct target of miR-100 was later confirmed SMRT/NCOR2 (silencing mediator of retinoid or thyroid hormone receptor-2/nuclear receptor corepressor 2) (Alrfaei et al. 2013).

Recently, many other miRNAs and their targets were described as involved in the regulation of proliferation, migration, and invasiveness in gliomas. Among them belong miR-326/NOB1 (Nin one binding protein) (Zhou et al. 2013); miR-125/PIAS3 (protein inhibitor of activated STAT, 3), which contributed to reduced STAT3 [signal transducer and activator of transcription 3 (acute-phase response factor)] transcriptional activity and subsequent decreased expression of MMP-2/9 (Shi et al. 2013); miR-138/ETH2 (methionine adenosyltransferase SAM2)–CDK4/6 (Cyclin-dependent kinase 4/6)–pRb (protein retinoblastoma 1)–E2F1 (E2F transcription factor 1) signal loop (Qiu et al. 2013); miR-495/CDK6 (Chen et al. 2013c); miR-149/AKT1 signaling (Pan et al. 2012); miR-330/SH3GL2 (SH3-domain GRB2-like 2) (Qu et al. 2012); miR-195/CCND3 (cyclin D3) (Zhang et al. 2012b); miR-34a/NOTCH1 (Li et al. 2011); miR-128/BMI1 (Godlewski et al. 2008); and miR-124, miR-137, miR-143, miR-145, and let-7 (Silber et al. 2008; Lee et al. 2011b; Koo et al. 2012).

4.3.2 MicroRNAs in Cell Cycle

Many miRNAs directly or indirectly target molecules such as cyclins, cyclin-dependent kinases, CDK regulators, and others, which are crucial for a progression of the cell through individual cell cycle checkpoints (Fig. 4.2).

The best-described miRNAs in gliomas with regard to the cell cycle are undoubtedly members of miR-34 family. Initially, overexpression of miR-34a in U251 GBM cells was described resulting in inhibition of cell growth and arrest in G0/G1 phase, which is *inter alia* accompanied by increased apoptosis. Moreover, authors supposed that this effect is due to the regulation of SIRT1 (sirtuin 1), a direct target of miR-34a (Luan et al. 2010). Two other studies confirmed c-Met (met proto-oncogene), NOTCH1, NOTCH2, and CDK6 also as direct targets of miR-34a and observed inhibition of G1/S progression after miR-34a artificial upregulation.

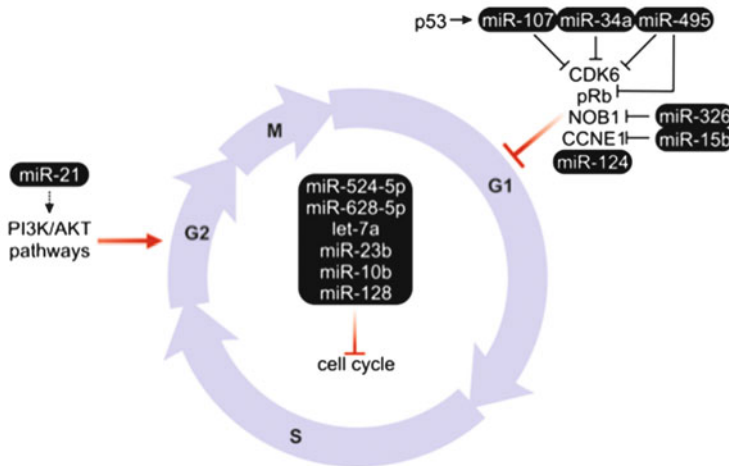


Fig. 4.2 MicroRNAs involved in regulation of glioma cell cycle. *CCNE1* cyclin E1; *CDK6* cyclin-dependent kinase 6; *NOB1* NIN1/RPN12 binding protein 1 homolog (*S. cerevisiae*); *p53* protein 53; *PI3K/AKT* phosphatidylinositol-4,5-bisphosphate 3-kinase/v-akt murine thymoma viral oncogene homolog; *pRb1* protein retinoblastoma 1. —| direct suppression; —> direct activation; ---> indirect activation

It seems that miR-34a regulates cell cycle via more important targets and, thus, presents a promising therapeutic target for brain tumors (Li et al. 2009a; Guessous et al. 2010b). CDK6 is confirmed as target molecule also for miR-495 and miR-107. Similar to miR-34a, cell cycle analysis revealed that upregulation of both miRNAs resulted in cell cycle arrest at the G1/S checkpoint. Interestingly, miR-107 inhibits also translation of NOTCH2 and its upstream regulator is p53 (protein 53) (Chen et al. 2013b). MiR-495 inhibits another crucial molecule of G1/S transition, tumor suppressor pRb, highlighting its potential in regulation of cell cycle (Chen et al. 2013c). Further, G1 cell cycle arrest was associated also with overexpression of miR-326, miR-15b, and miR-124. Nevertheless, the mechanisms seem to be different. While miR-326 targets NOB1, miR-15b decreases expression of downstream *CCNE1* gene encoding cyclin E1 (Xia et al. 2009; Zhao et al. 2013b; Zhou et al. 2013).

But miRNAs are not involved just in the disruption of G1/S transition; it seems that miR-21 decreases G2/M transition. Gwak et al. showed that transfection of malignant glioma cells by anti-miR-21 resulted in the increased transition at this phase. The mechanism of miR-21 modulation of G2/M transition is probably similar to its regulation of proliferation, through the activation of the PI3K/AKT pathway (Gwak et al. 2012).

Finally, the ability to arrest a cell cycle progression in gliomas is linked also with miR-524-5p, miR-628-5p, let-7a, miR-23b, miR-10b, and miR-128 (Zhang et al. 2009; Gabriely et al. 2011; Jiang et al. 2013; Li et al. 2013c; Wang et al. 2013c).

4.3.3 MicroRNAs in Apoptosis

MiRNAs are important regulators of apoptotic pathways. Alterations in functioning of these apoptotic miRNAs are frequently observed also in the glial tumors (Fig. 4.3). One of the most important regulatory mechanisms of apoptosis in glioma cells is cross talk between TGF- β (transforming growth factor, beta) and p53 signaling, which are regulated by the well-known onco-miR, miR-21. Papagiannakopoulos et al. reported that p53, TGF- β , and mitochondrial apoptotic networks are derepressed in response to miR-21 knockdown. They published a panel of genes, which are involved in particular pathways and are simultaneously modulated by miR-21 treatment. Some of these genes were subsequently predicted to be direct targets of miR-21 that can stabilize p53 protein levels by interfering with MDM2 (MDM2 oncogene, E3 ubiquitin protein ligase) and/or act as p53 transcriptional cofactors (Papagiannakopoulos et al. 2008; Sana et al. 2011). Inhibition of miR-21 increased also endogenous levels of PDCD4 in human glioma cell lines and activated caspases 9/3, which may be mediated by modulating multiple potential target genes, such as *TIMP3* (tissue inhibitor of matrix metalloproteinase 3) (Chen et al. 2008; Zhou et al. 2010b; Sana et al. 2011). The upregulation of PDCD4 and caspase 3 is in contrast to the low level of miR-21 and inactivation of TGF- β 1/SMAD signaling, which are the critical upstream regulators of miR-21 in dose- and time-dependent manner in human GBM cell line U251 (Wang et al. 2012a).

Other direct targets of miR-21 involved in apoptosis were confirmed molecules ANP32A (acidic (leucine-rich) nuclear phosphoprotein 32 family, member A) and SMARCA4 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4). In A172 GBM cells, enhanced ANP32A expression compensated for the positive effects of anti-miR-21 treatment on cell viability and apoptosis (Schramedei et al. 2011). MiR-21 silencing also enhances apoptosis of GBM cells also after treatment by the antiangiogenic drug sunitinib and alkylation agent temozolomide (TMZ) (Zhang et al. 2012c; Costa et al. 2013). In relation to the enhancement of chemosensitivity was described also miR-155. This miRNA indicated oncogenic character and its silencing led to the elevation of apoptosis after taxol treatment via EAG1 (potassium voltage-gated channel, subfamily H (eag-related), member 1) expression (Meng et al. 2012).

Interestingly, miR-211 overexpression and MMP-9 treatments led to the activation of the intrinsic mitochondrial/caspase-9/3-mediated apoptotic pathway in both glioma cells and GSCs (Asuthkar et al. 2012). MiR-34a was identified also as regulator of TGF- β signaling in GBM (Genovese et al. 2012). This miRNA was suggested to be promoter of mitochondrial apoptosis in several cancers including gliomas (Li et al. 2011; Sasaki et al. 2012; Bienertova-Vasku et al. 2013). Therefore, it is probable that miR-34a regulates apoptosis in glioma also through the TGF- β signaling pathway.

There are also other miRNAs described as regulators of apoptosis in glioma cells. Overexpression of miR-326 in human glioma cell lines A172 and U373 caused cell cycle arrest at the G1 phase, delayed cell proliferation, and mainly enhanced apoptosis (Zhou et al. 2013). Interestingly, miR-326 targets PKM2

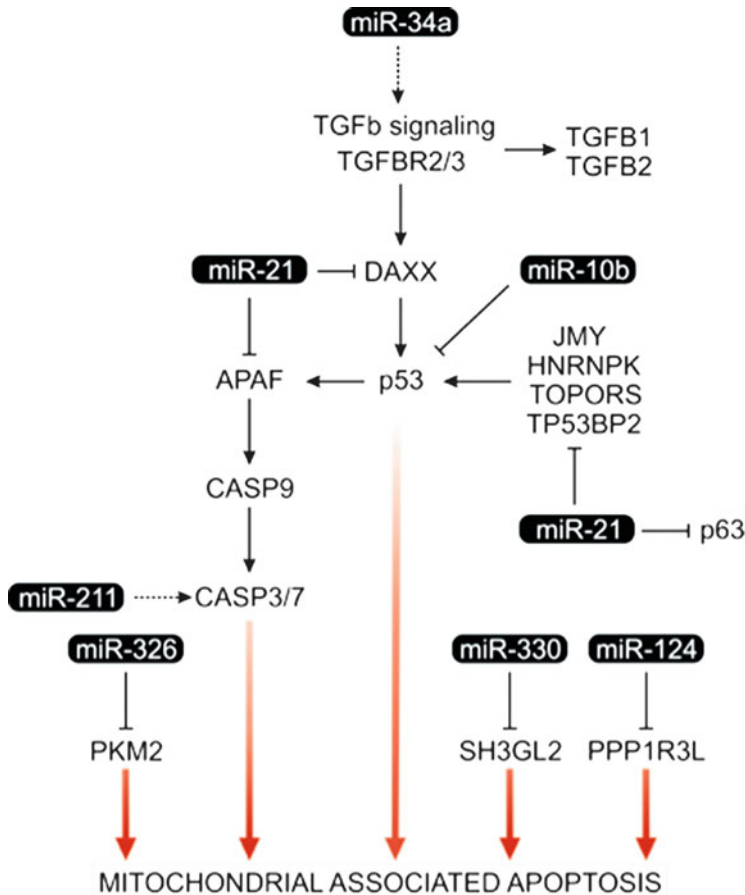


Fig. 4.3 MicroRNAs involved in regulation of glioma mitochondrial associated apoptosis. *APAF* apoptotic peptidase activating factor; *CASP3/7/9* caspase 3/7/9, apoptosis-related cysteine peptidase; *DAXX* death-domain associated protein; *HNRNPK* heterogeneous nuclear ribonucleoprotein K; *JMY* junction mediating and regulatory protein, p53 cofactor; *p53* protein 53; *p63* protein 63; *PKM2* pyruvate kinase 2; *PPP1R3L* an inhibitory member of the apoptosis-stimulating protein of p53 family (IASPP); *SH3GL2* SH3-domain GRB2-like 2; *TGFβ1/2* transforming growth factor, beta 1; *TGFβR2/3* transforming growth factor, beta receptor 2/3; *TOPORS* topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase; *TP53BP2* tumor protein p53 binding protein, 2. —| direct suppression; —> direct activation; -.-> indirect activation

(pyruvate kinase M2), which plays a key role in cancer cell metabolism. This could be a mechanism how miR-326 regulates glioma cell survival (Kefas et al. 2010). MiR-10b suppresses many tumor suppressors including p53, FOXO3 (forkhead box O3), CYLD [cylindromatosis (turban tumor syndrome)], PAX6 (paired box 6), PTCH1 (patched 1), HOXD10, and NOTCH1. Hence, this miRNA shows pleiotropic feature and regulates many key cellular processes (Lin et al. 2012) and its downregulation; it is not expressed in human brain and strongly upregulated

in both low-grade and high-grade gliomas and reduces glioma cell growth through cell cycle arrest and activation of apoptosis (Gabriely et al. 2011). MiR-124 regulates apoptosis and other cell processes via PPP1R3L (Zhao et al. 2013b). Similar pleiotropic nature has also onco-miR miR-330 targeting SH3GL2 (Qu et al. 2012).

4.3.4 MicroRNAs in Angiogenesis

Angiogenesis is essential for many physiological processes as well as for tumor development. It can be triggered by extracellular signals such as vascular endothelial growth factors and by genetic alterations such as activation of oncogenes (Sternlicht et al. 1999; Folkman 2006; Zheng et al. 2013). In gliomas, aberrant activation of angiogenic signaling pathways EGFR/PI3K/AKT, NF- κ B, VEGF (vascular endothelial growth factor), and PDGF (platelet-derived growth factor) was repeatedly described (Kozomara and Griffiths-Jones 2011; Mizoguchi et al. 2012).

Glioma is one of the first cancers in which angiogenesis was found to be a key phenotypic feature to disease progression. Indeed, GBM is one of the most highly vascularized human cancers. It is well recognized that the progression of human glioma is angiogenesis dependent. Accumulated evidence has shown that high vascular density and overexpression of angiogenic factors correlate with poor prognosis of glioma patients (Mizoguchi et al. 2012).

Recently, several miRNAs were described to be involved in glioma angiogenesis regulation (Fig. 4.4). In addition to miR-30e* and miR-182 activating NF- κ B signaling and subsequent expression of downstream angiogenic factors such as VEGF-C and MMPs (Mizoguchi et al. 2012), tumor-suppressive miR-205 can specifically suppress expression of the key angiogenic factor, VEGF-A. The effect of the miR-205 ectopic expression has not only anti-angiogenic impact, but it is accompanied by the induction of apoptosis, cell cycle arrest, impairing of cell viability, clonability, and invasiveness (Yue et al. 2012). Angiogenic function was supposed also for miR-15b and miR-152, but subsequent evaluation of tube formation in cultured endothelial cells with culture supernatant from 9L cells, rat glial cell line derived from gliosarcoma, treated with these miRNAs revealed that only miR-15b significantly reduced capillary-like tube formation. Authors have suggested that this effect is caused by NRP2 (neuropilin 2), which was confirmed to be a direct target of miR-15b. NRPs are receptors for the SEMA (class-3 semaphorin) family of axon guidance molecules and also for VEGF family of angiogenic factors. VEGF–NRP interactions promote developmental angiogenesis and also metastases (Geretti and Klagsbrun 2007; Zheng et al. 2013).

Glioma cells and angiogenic growth factors elevate the level of miR-296 in primary human brain microvascular endothelial cells in culture. Subsequently, growth factor-induced miR-296 significantly contributes to angiogenesis by directly targeting the HGS (hepatocyte growth factor-regulated tyrosine kinase substrate) leading to reduced HGS-mediated degradation of the VEGFR2 (vascular endothelial growth factor receptor 2) and PDGFR β (platelet-derived growth factor

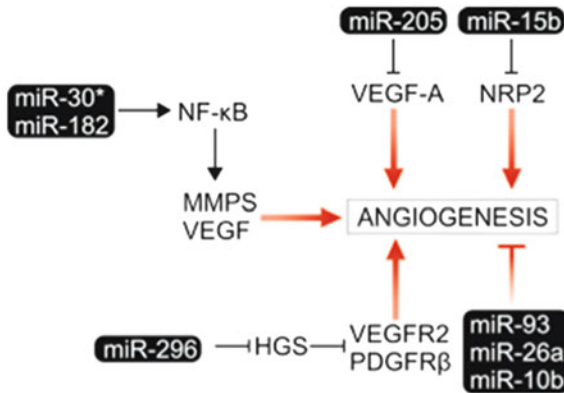


Fig. 4.4 MicroRNAs involved in regulation of glioma angiogenesis. *HGS* hepatocyte growth factor-regulated tyrosine kinase substrate; *MMPs* matrix metalloproteinases; *NF-κB* nuclear factor of kappa light polypeptide gene enhancer in B-cells; *NRP2* neuropilin 2; *PDGFRB* platelet-derived growth factor receptor, beta polypeptide; *VEGF-A* vascular endothelial cell growth factor A; *VEGFR2* VEGF kinase insert domain receptor (a type III receptor tyrosine kinase). —| direct suppression; —> direct activation; ---> indirect activation

receptor, beta). Furthermore, inhibition of miR-296 reduces angiogenesis in tumor xenografts in vivo (Wurdinger et al. 2008). Similarly, when miR-93-overexpressing U87 glioma cells were cocultured with endothelial cells, they supported endothelial cell spreading, growth, migration, and tube formation. In vivo studies revealed that miR-93-expressing cells induced blood vessel formation allowing blood vessels to extend to tumor tissues in high densities. This effect is explained by suppressing, at least in part, integrin-β8 expression (Fang et al. 2011).

MiR-26a has been described above as a regulator of proliferation. In addition, forced expression of miR-26a in glioma significantly increased tumor growth and angiogenesis in vivo, while reduced expression of this miRNA played opposite role (Qian et al. 2013). Functionally pleiotropic miR-10b is able to regulate angiogenicity in tumor cells resembling mesenchymal subtype of GBM (Lin et al. 2012).

4.3.5 MicroRNAs in Immune Response

Interferons (IFNs) are cytokines released by lymphocytes that have antiviral, antiproliferative, and immunomodulatory effects. They are connected with the JAK-STAT (Janus kinase-Signal Transducer and Activator of Transcription) signaling pathway and allow communication between cells to trigger protective defenses of the immune system leading to eradication of affected cells. Therefore, deregulation of JAK-STAT cascade is the key step to mediating immunosuppression in the tumor microenvironment (Platanias 2005; Sana et al. 2011). Recently,

the possibility was investigated if IFN- β may induce or reduce cellular miRNAs in human gliomas. Analysis of IFN- β treatment on miR-21 expression in glioma cells and intracranial glioma xenografts revealed that systemic delivery of this cytokine markedly reduced the level of miR-21 in glioma cells. In contrast, the addition of the STAT3-specific inhibitor increased the level of miR-21 (Ohno et al. 2009; Sana et al. 2011). Another study revealed miR-221 and miR-222 as possible regulators of IFN pathways. Zhang et al. found that the IFN- α signaling pathway is the most significant pathway modulated by genes with the different expression after knock-down of miR-221 and miR-222. The authors showed that STAT1 and STAT2 expression and phosphorylation were upregulated in U251 cells with silenced miR-221/222. Tyrosine phosphorylation of STAT1 and STAT2 was present in the nucleus after repression of the same miRNAs. These data illustrate a mechanism of STAT1/2 upregulation under the transcriptional control of IFN- α signaling after knockdown of miR-221/222 cluster in U251 glioma cells (Zhang et al. 2010a; Sana et al. 2011).

Interestingly, on the basis of miRNA expression in gliomas using tissue microarrays, in situ hybridization, and molecular modeling, miR-124 was identified as another candidate for modulating STAT3 signaling pathway. MiR-124 is absent in all grades and pathologic types of gliomas. Upon replacement of miR-124 in GSCs (glioma stem cells), the STAT3 pathway was inhibited, and miR-124 reversed GSC-mediated immunosuppression of T-cell proliferation and induction of Foxp3 (forkhead box P3) regulatory T cells (Treg). Treatment of T cells from immunosuppressed GBM patients with miR-124 induced remarkable effector response including upregulation of IL-2 (interleukin 2), IFN- γ , and TNF- α (tumor necrosis factor, alpha). Both systemic administration of miR-124 and adoptive miR-124-transfected T-cell transfers caused strong anti-glioma therapeutic effects and enhanced effector responses in the local tumor microenvironment in vivo. These findings highlight the potential application of miR-124 as a novel immunotherapeutic agent for gliomas (Shi et al. 2013).

4.3.6 MicroRNAs in Glioma Stem Cells

During the last years there is an increasing interest in the small population of glioma cells, later termed as glioma stem cells (GSCs) or glioma initiating cells (GICs), which have the ability to self-renew (Koshkin et al. 2013). Conventional therapies target the cells that rapidly divide, while the benefit of GICs is to divide slowly but infinitely. These properties enable them to initiate new tumors and also to serve as a source of recurrence (Martin et al. 2013). These cells, which maintain stem-like phenotype, show generally much more resistance to radiation or chemotherapy in comparison to “normal” glioma cells (Wang et al. 2010; Yamada and Nakano 2012). Similar to normal stem cells, GSCs express CD133 (prominin 1), NESTIN, OCT4 (POU class 5 homeobox 1), SOX2 (SRY (sex determining region Y)-box 2), and NANOG (Nanog homeobox) markers and are capable of self-renewal as well as they have the ability to initiate neurosphere growth in vitro and generation of highly

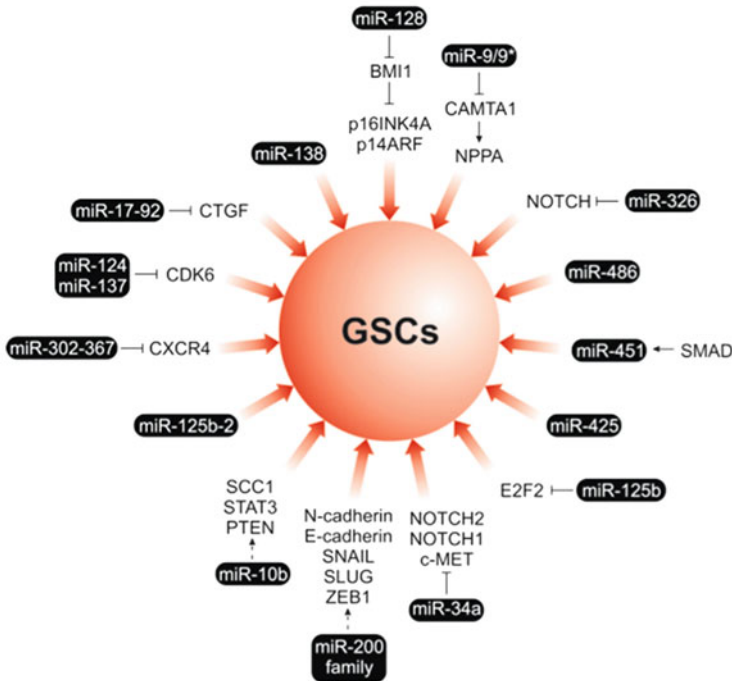


Fig. 4.5 MicroRNAs involved in regulation of glioma stem cells. *BMI1* BMI1 polycomb ring finger oncogene; *c-MET* met proto-oncogene; *CAMTA1* calmodulin binding transcription activator 1; *CDK6* cyclin-dependent kinase 6; *CTGF* connective tissue growth factor; *CXCR4* chemokine (C-X-C motif) receptor 4; *E2F2* E2F transcription factor 2; *NPPA* natriuretic peptide A; *p14ARF* cyclin-dependent kinase inhibitor 2A; *p16INK4A* cyclin-dependent kinase inhibitor 2A; *PTEN* phosphatase and tensin homolog; *SCC1* protein tyrosine phosphatase; *SLUG* snail family zinc finger 2; *SMAD* SMAD family member; *SNAIL* snail family zinc finger 1; *STAT3* signal transducer and activator of transcription 3 (acute-phase response factor); *ZEB1* zinc finger E-box binding homeobox 1. —| direct suppression; —> direct activation; ---> indirect activation

malignant tumor in NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice (Altaner 2008; Chen et al. 2010b). There is increasing amount of evidence suggesting involvement of miRNAs in the regulation of stem-like phenotype of GSCs (Fig. 4.5).

Two independent research groups found out that miR-128 inhibits self-renewal and proliferation capacities of GSCs through the direct targeting of *BMI1* (Godlewski et al. 2008; Fu et al. 2013), which negatively regulates expression of tumor-suppressor proteins p16INK4a (cyclin-dependent kinase inhibitor 2A) and p14ARF (*CDKN2A*; cyclin-dependent kinase inhibitor 2A). These two molecules subsequently regulate cyclin D and p53 leading to the cell cycle arrest (Siddique and Saleem 2012). Fu et al. described capability of miR-200 family to suppress an epithelial–mesenchymal transition (EMT) in GSCs via E-cadherin, N-cadherin, *SNAIL*, *SLUG*, and *ZEB1* (zinc finger E-box binding homeobox 1)

(Fu et al. 2013). It was also discovered that miR-200c is involved in the regulation of BMI1 in breast cancer and, thus, this miRNA could function analogically to miR-128 in GSCs (Siddique and Saleem 2012).

Chan et al. published data showing miR-138 being an important regulator of self-renewal, proliferation, and apoptosis in GSCs. Interestingly, this miRNA did not affect healthy neural stem cells. This fact is remarkable accordingly to potential use of miR-138 in therapy (Chan et al. 2012). The similar effects indicated also miR-10b, the inhibition of which decreased proliferation, migration, and invasiveness in vitro and, moreover, the ability of GSCs to initiate tumorigenesis in vivo (Guessous et al. 2013). Oncogenic properties have also miR-9/9* that inhibit expression tumor suppressor CAMTA1 (calmodulin-binding transcription activator 1) and thus support tumorigenic potential of CD133 positive cells. CAMTA1 activates transcription of NPPA (antiproliferative peptide), protein with proven antiproliferative effects in GBM. Furthermore, expression levels of both CAMTA1 and NPPA correlate with prognosis and overall survival of GBM patients (Schraivogel et al. 2011).

On the contrary, miR-34a was observed as tumor-suppressive miRNA in GBM. This molecule targets genes involved in tumorigenesis as well as in regulation of GSCs (c-MET, NOTCH1, NOTCH2). It was demonstrated that miR-34a has significantly lower expression in glioma cells and, further, decreases cell proliferation, migration, invasiveness, and viability when replaced in vitro (Li et al. 2009a). This replacement approach led in GSCs to the induction of apoptosis and differentiation, which was connected with significant reduction of stem cell markers CD133 and nestin (Guessous et al. 2010a). Another regulator of NOTCH signaling is miR-326 that also decreases viability, invasiveness, and tumorigenic potential in GBM when its levels are upregulated (Kefas et al. 2009). MiR-125 is another molecule involved in the regulation of neural differentiation (Le et al. 2009), which is downregulated in GBM tissue and CD133 positive GBM cells. Ectopic expression of miR-125 led to the inhibition of proliferation and sphere formation, to cell cycle arrest in G0–G1 phase, and to the decreased expression of stemness markers indicating cell differentiation. Further, E2F2 (E2F transcription factor 2), critical regulator of cell cycle, is the direct target of miR-125b (Wu et al. 2012). Moreover, miR-124 and miR-137 are involved in a regulation of the cell cycle progression through G1 phase into early S phase in GSCs via inhibition of CDK6. This results in decreased proliferation and enhanced differentiation (Silber et al. 2008). In another study was observed that miR-125b-2 is overexpressed in initial GBMs as well in relapsed tumors after concomitant RT/TMZ (radiochemotherapy with temozolomide) therapy and, thus, this miRNA indicates oncogenic character. Increased levels of miR-125b-2 were also detected in CD133 positive GBM cells. Inhibition of this miRNA leads to the induction of mitochondrial apoptosis after TMZ treatment in CD133 positive cells Shi et al. 2012.

As an important regulators of glioblastoma tumor initiating cells (GICs; this term is sometimes used by authors, who decline to use the term glioma stem cell) have been described also members of cluster miR-302-367. This cluster is able to suppress the self-renewal, infiltration, and proliferation potential of GICs through a

repression of CXCR4 (chemokine C-X-C motif, receptor 4) leading to the suppression of SHH-GLI-NANOG signaling pathway (Card et al. 2008; Zbinden et al. 2010; Fareh et al. 2012). Similarly, another cluster miR-17-92 regulates neurosphere formation, differentiation, proliferation, and apoptosis in vitro in GICs. Supposed mediator of these effects is the gene CTFG (connective tissue growth factor), a direct target of this cluster (Ernst et al. 2010). Global expression analysis of miRNAs revealed changes during differentiation of GICs, including overexpression of miR-21, miR-29a, miR-29b, miR-221, and miR-222 and downregulation of miR-93 and miR-106a. Functional studies showed that miR-21 overexpression in GICs induced comparable cell differentiation features and targeted SPRY1 [sprouty homolog 1, antagonist of FGF signaling (*Drosophila*)] mRNA, which encodes for a negative regulator of neural stem cell differentiation. In addition, miR-221 and miR-222 inhibition in differentiated cells restored the expression of stem cell markers while reducing differentiation markers. Finally, miR-29a and miR-29b targeted MCL1 [myeloid cell leukemia sequence 1 (BCL2-related)] mRNA in GICs and increased apoptosis (Aldaz et al. 2013).

Analysis of the expression profiles of both CD133 positive and negative GBM cells revealed several differentially expressed miRNAs including miR-451, miR-486, and miR-425. Ectopic expression of these miRNAs led to the inhibition of cell growth and neurosphere formation in GBM cells. As positive regulator of miR-451 was subsequently revealed SMAD (Gal et al. 2008). Another analysis showed that miR-153 expression was downregulated in GBM tissues relative to normal brain tissues and in CD133 positive cells relative to CD133 negative cells (Zhao et al. 2013a).

4.4 MicroRNAs as Biomarkers in Gliomas

Recent research suggests that deregulation of miRNAs is involved in initiation and progression of many cancers, including gliomas, and that miRNAs hold great potential as future diagnostic, prognostic, and predictive biomarkers in cancer. The rationale of this potential is mainly based on the miRNAs' increased stability when compared to mRNAs and the ability of a single miRNA molecule to affect numerous targets, oncogenes and tumor-suppressor genes including (Hermansen and Kristensen 2013).

4.4.1 Diagnostic Biomarkers

Study of miRNA expression profiles in tumor tissue of gliomas of different grades and their comparison to miRNA profiles in non-tumoral brain tissue is not only the first step in the determination of miRNAs involved in glioma pathogenesis, but also important approach enabling new level of glioma molecular taxonomy, which is not based on morphology but on concrete molecular alterations which are present in individual tumors. This molecular classification has potential to be more accurate in

Table 4.1 MicroRNAs significantly deregulated in glioma tissue, which were observed in at least two independent studies

miRNA study	Chan	Sasayama	Conti	Wang	Ciafrè	Slaby	Silber	Rao	Others
miR-17								↑	↑
miR-19a								↑	↑
miR-19b								↑	↑
miR-21	↑		↑	↑	↑	↑	↑	↑	
miR-25					↑			↑	
miR-92b		↑						↑	
miR-106b		↑						↑	
miR-125b					↑	↑			
miR-130a					↑			↑	
miR-155							↑	↑	
miR-182				↑				↑	
miR-335				↑					↑
miR-10b					↑				↓
miR-138	↑								↓
miR-221			↑		↑	↓			
miR-34a									2x ↓
miR-128a					↓	↓	↓		
miR-132							↓	↓	
miR-181a					↓	↓			
miR-181b			↓		↓	↓			
miR-181c					↓	↓			
miR-198	↓							↓	
miR-219-5p				↓				↓	
miR-329		↓						↓	
miR-338-3p				↓				↓	
miR-483-5p				↓				↓	

↑ upregulated in gliomas, ↓ downregulated in gliomas

stratification of the patients accordingly to their prognosis and therapeutic response in comparison to conventional histopathological classification and open new diagnostic possibilities for glioma patients. MiRNAs which were repeatedly found to be differentially expressed between glioma tumor tissue and non-tumoral brain tissue are summarized in Table 4.1.

The first work aiming miRNA profiling in glioblastoma tissue was published by Ciafre et al. (2005). These authors analyzed 254 miRNAs in nine paired GBM and non-tumor brain tissue samples. The findings showed that nine miRNAs (miR-10b, miR-130a, miR-221, miR-125b-1, miR-125b-2, miR-9-2, miR-21, miR-25, and miR-123) were upregulated and four miRNAs (miR-128a and miR-181a/b/c) downregulated in GBM. The greatest expression changes were observed in miR-221, miR-128a, and miR-181a/b/c (Ciafre et al. 2005). A similar analysis was performed by Chan et al., which compared three primary high-grade gliomas with eight samples of fetal and adult brain tissues. In this study, five miRNAs

(miR-21, miR-138, miR-347, miR-135, and miR-291-5) were significantly overexpressed and three miRNAs (miR-198, miR-188, and miR-202) showed lower expression levels in tumor tissue. MiR-21 was almost nine times more expressed in GBM compared to control samples (Chan et al. 2005). Silber et al. analyzed 192 matured miRNAs in four anaplastic astrocytomas (grade III), four GBMs (grade IV), and four non-tumor tissues obtained from surgically resected tissue of temporal lobes of epileptic patients. Statistical analysis revealed 13 downregulated and three upregulated miRNAs in GBM compared with the non-tumor tissue. Furthermore, only six deregulated miRNAs were observed between grade III and IV gliomas (Silber et al. 2008).

Similar study was published comparing global expression profiles of miRNAs in 39 gliomas (13 primary and 13 secondary GBMs, 13 anaplastic astrocytomas) and seven normal brain tissues and revealed 55 upregulated and 29 downregulated miRNAs in glioma tissue. Moreover, 67 differentially expressed miRNAs were identified by a comparison of anaplastic astrocytomas and GBMs. The comparison of primary and secondary GBMs revealed seven differentially expressed miRNAs. Several miRNAs significantly differed also between sequentially progressive astrocytomas (anaplastic astrocytomas and secondary GBM) and primary GBMs (21 miRNAs), anaplastic astrocytomas and primary GBMs (76 miRNAs), as well as anaplastic astrocytomas and secondary GBMs (68 miRNAs) (Rao et al. 2010). Indeed, D'Urso et al. found that a set of 10 miRNAs is able to classify primary and secondary GBMs (D'Urso et al. 2012).

Skalsky and Cullen investigated miRNA expression profiles in six GBMs and three samples of non-tumor brain tissues using high-throughput sequencing technology. They published 68 differentially expressed miRNAs between both examined sets ($p < 0.05$, Student's *t*-test). Nevertheless, when they applied the standard FDR (false discovery rate)-adjusted p -value < 0.05 cutoff, only nine miRNAs (miR-124, miR-10b*, miR-139-5p, miR-7, miR-10b, miR-132, miR-95, miR-543, miR-7d) were identified that met the criteria. Given the much broader dynamic range of deep sequencing compared to finite platforms such as miRNA microarrays or real-time PCR (Polymerase Chain Reaction) arrays, they therefore used an FDR cutoff of $p < 0.1$. Under these conditions 12 more miRNAs (miR-323-3p, miR-128, miR-139-3p, miR-598, miR-103a, miR-103b, miR-873, miR-891a, miR-487b, miR-323b-3p, miR-138-1*, miR-93) met the criteria (Skalsky and Cullen 2011).

Sasayama et al. analyzed three paired samples of GBM and non-tumor brain tissues. They found that five miRNAs (miR-10b, miR-21, miR-183, miR-92b, and miR-106b) were upregulated and five miRNAs (miR-302c*, miR-379, miR-329, miR-134, and miR-369-3p) were downregulated in GBMs (Sasayama et al. 2009). Wang et al. also analyzed miRNA expression profiles in three GBMs and three paired non-tumor adjacent tissues. They identified 91 miRNAs, whose expressions were at least twofold changed between both sets of samples. Interestingly, an expression of miR-483-5p was almost 100-fold decreased in tumors (Wang et al. 2012b). Another study validated expression of eight selected miRNAs in 10 GBM tissues in comparison to the four non-tumor samples of brain tissues obtained from epileptic patients. MiR-21 and miR-221 showed higher and

miR-181b lower expression levels in tumor samples (Conti et al. 2009). Slaby et al. compared expressions of eight selected miRNAs in GBMs and non-tumor brain tissues obtained from surgeries of arteriovenous malformations (AVM). Results showed that while miR-21 and miR-125b were overexpressed in GBMs, miR-128a, miR-181a/b/c, and miR-221/222 were downregulated in tumors (Slaby et al. 2010). Other studies show that miR-31 (Hua et al. 2012), miR-205 (Yue et al. 2012), miR-124a (Fowler et al. 2011), and miR-34a (Li et al. 2011) have lower expression levels in GBM in comparison with normal brain tissue.

Malzkorn et al. analyzed miRNA expression profiles among different grades of gliomas (low-grade astrocytomas vs. anaplastic astrocytomas vs. secondary GBMs). Authors studied expression profiles of 157 miRNAs in patients affected by low-grade astrocytomas (grade II) that gradually progressed to secondary GBMs (grade IV). It was found that 12 miRNAs (miR-9, miR-15a, miR-16, miR-17, miR-19a, miR-20a, miR-21, miR-25, miR-28, miR-130b, miR-140, and miR-210) are upregulated and two miRNAs (miR-184 and miR-328) are downregulated dependently on the grade of glioma (Malzkorn et al. 2010).

There are also many studies characterizing expression pattern of only one miRNA in gliomas and their different clinicopathological features. From these studies it implies that miR-34a, miR-203, miR-326, and miR-375 are reduced in tumors (Chang et al. 2012; Gao et al. 2013; He et al. 2013; Wang et al. 2013b) and miR-17, miR-19a/b, miR-224, miR-335, and miR-372 are overexpressed in gliomas (Jiang et al. 2012; Lu et al. 2012, 2013; Jia et al. 2013; Li et al. 2013a) compared to non-tumor brain tissues. Moreover, most of these miRNAs were significantly correlated with grading of gliomas.

4.4.2 Prognostic and Predictive Biomarkers

One of the main aims in glioma research is the discovery of highly sensitive prognostic and predictive biomarkers enabling stratification of the patients accordingly to their risk of progression and predicted therapeutic response. The importance of this challenge increases with the advent of new therapeutic possibilities in the treatment of gliomas, e.g., TMZ, bevacizumab, and cilengitide (Chamberlain 2011). MiRNAs with potential as prognostic and/or predictive biomarkers are summarized in Table 4.2.

Srinivasan et al. analyzed 10 selected miRNAs in 222 GBM patients. The aim of this study was to find differently expressed miRNAs between short-time and long-time surviving GBM patients. Tumor samples of the patients with long-time OS (overall survival) showed higher expression levels of protective miRNAs (miR-20a, miR-106a, miR-17-5p). Conversely, overexpression of oncogenic miRNAs (miR-31, miR-221, miR-222, miR-148a, miR-146b, miR-200b, miR-193a) was observed in tumors of patients with short-time OS (Srinivasan et al. 2011). Further, Niyazi et al. performed retrospective study analyzing 1,100 miRNAs in 35 GBM patients, who underwent adjuvant concomitant chemoradiotherapy. Subsequently, 30 miRNAs, which mostly differ between short- and long-time surviving patients,

Table 4.2 MicroRNAs with prognostic and/or predictive significance in gliomas

MiRNA	Glioma (WHO)	Prognostic and/or predictive significance	References
let-7a	IV	Correlation with survival (+)	Niyazi et al. (2011)
miR-106a	IV	Correlation with survival (+)	Srinivasan et al. (2011)
miR-1260	IV	Correlation with survival (+)	Niyazi et al. (2011)
miR-1305	IV	Correlation with survival (+)	Niyazi et al. (2011)
miR-146b	IV	Correlation with survival (–)	Srinivasan et al. (2011)
miR-148	IV	Correlation with survival (–)	Srinivasan et al. (2011)
miR-17	I–IV	Correlation with survival (–)	Srinivasan et al. (2011)
miR-17-5p	IV	Correlation with survival (+)	Srinivasan et al. (2011)
miR-181b	IV	Association with poor response to RT/TMZ	Slaby et al. (2010)
miR-181c	IV	Association with poor response to RT/TMZ	Slaby et al. (2010)
miR-193a	IV	Correlation with survival (–)	Srinivasan et al. (2011)
miR-195	IV	Correlation with survival (+)	Lakomy et al. (2011)
miR-196a	III–IV	Correlation with survival (–)	Ujifuku et al. (2010)
miR-196b	IV	Correlation with survival (+)	Lakomy et al. (2011)
miR-196b	III–IV	Correlation with survival (–)	Ujifuku et al. (2010)
miR-200b	IV	Correlation with survival (–)	Srinivasan et al. (2011)
miR-203	I–IV	Correlation with survival (+)	He et al. (2013)
miR-20a	IV	Correlation with survival (+)	Srinivasan et al. (2011)
miR-21	I–IV	Correlation with survival (–)	Hermansen et al. (2013)
miR-214	I–IV	Correlation with survival (+)	Wang et al. (2014)
miR-221	IV	Correlation with survival (–)	Srinivasan et al. (2011)
miR-222	IV	Correlation with survival (–)	Srinivasan et al. (2011)
miR-224	I–IV	Correlation with survival (–)	Lu et al. (2013)
miR-31	IV	Correlation with survival (–)	Srinivasan et al. (2011)
miR-3163	IV	Correlation with survival (–)	Niyazi et al. (2011)
miR-326	I–IV	Correlation with survival (+)	Wang et al. (2013b)
miR-335	I–IV	Correlation with survival (–)	Jiang et al. (2012)
miR-34a	I–IV	Correlation with survival (+)	Gao et al. (2013)
miR-372	I–IV	Correlation with survival (–)	Li et al. (2013a)
miR-375	I–IV	Correlation with survival (+)	Chang et al. (2012)
miR-539	IV	Correlation with survival (+)	Niyazi et al. (2011)
miR-650	I–IV	Correlation with survival (–)	Sun et al. (2013)
miR-9	I–IV	Correlation with survival (–)	Wu et al. (2013)

were selected for the following analysis. However, only five miRNAs were significantly deregulated: miR-3163 (fold change (FC) = 2.0, $p = 0.05$), miR-539 (FC = 0.5, $p = 0.001$), miR-1305 (FC = 0.5, $p = 0.05$), miR-1260 (FC = 0.5, $p = 0.03$), and let-7a (FC = 0.3, $p = 0.02$). Nevertheless, an application of all

30 miRNAs classified tested patients into two groups according to overall survival. The miRNA pattern and the prognostic power were both independent of the MGMT (O-6-methylguanine-DNA methyltransferase) methylation status. When performing a multivariate analysis for all patients including the factors MGMT status, miRNA pattern, adjuvant TMZ, age category, and RPA (recursive partitioning analysis) class, it turns out that only adjuvant TMZ remains a prognostic factor ($p = 0.01$) and MGMT status and miRNA pattern lose their prognostic significance ($p = 0.17$ and $p = 0.22$) (Niyazi et al. 2011).

Recent study published by Slaby et al. described significant negative impact of miR-181c and miR-181b on the response to the concomitant chemoradiotherapy with TMZ (Slaby et al. 2010). Lakomy et al. analyzed expression of eight miRNAs (miR-21, miR-128a, miR-181c, miR-195, miR-196a, miR-196b, miR-221, and miR-222) in GBM patients treated with concomitant chemoradiotherapy with TMZ and results subsequently correlated with MGMT status and clinical data. It was found that miR-195 ($p = 0.0124$) and miR-196b ($p = 0.0492$) had a positive impact on overall survival of the patients. Moreover, combination of miR-181c and miR-21 enabled to identify patients, which progressed in the 6 months after diagnosis (sensitivity 92 %, specificity 81 %, $p < 0.0001$) (Lakomy et al. 2011). These results are partially conflicting with conclusions of Ujifuku et al., who identified miR-195, miR-455-3p, and miR-10a* to be three most increased miRNAs in the TMZ-resistant cell lines. Moreover, miR-195 silencing led to the increased sensitivity to TMZ in these cells (Ujifuku et al. 2010). Similarly, Guan et al. described significant relationship between high expression of miR-196a/b and a worse prognosis of GBM and anaplastic astrocytoma patients (Guan et al. 2010). However, it is interesting that miR-195 and miR-196b have the same impact on prognosis in colorectal, hepatocellular, and adrenocortical carcinomas as described by Lakomy et al. (Soon et al. 2009; Xu et al. 2009; Liu et al. 2010; Wang et al. 2012c).

Wang et al. evaluated the prognostic value of miR-214 in overall survival of 108 glioma patients (WHO I—18, WHO II—12, WHO III—32, WHO IV—46). The overall survivals of patients, whose tumors expressed low level of miR-214, were significantly shorter than those with high level of miR-214 ($p < 0.001$). Moreover miR-214 was significantly downregulated compared to 20 normal brain tissues ($p = 0.001$) (Wang et al. 2014). Similarly, miR-34a, miR-203, miR-326, and miR-375 were reduced in tumors and their lower expression levels were significantly associated with worse progression-free survival and overall survival of glioma patients. Furthermore, multivariate Cox regression analyses indicated that all these miRNAs have been independent prognostic factors (Chang et al. 2012; Gao et al. 2013; He et al. 2013; Wang et al. 2013b).

On the contrary, miR-9 expression in 128 glioma tissues (WHO I—18, WHO II—14, WHO III—38, WHO IV—58) was significantly higher in comparison to 10 corresponding non-neoplastic brain tissues ($p < 0.001$). The increased expression of miR-9 was more frequently observed in the tissue of high-grade gliomas ($p = 0.001$). The expression levels of miR-9 in glioma tissues with low Karnofsky performance score (KPS) were also significantly higher than those with high KPS

($p = 0.008$). Moreover, the overall survival of glioma patients with high miR-9 expression was obviously lower than that with low miR-9 expression ($p < 0.001$). Multivariate analysis further showed that high miR-9 expression was an independent prognostic factor for overall survival in glioma patients ($p = 0.01$). More importantly, the subgroup analyses indicated that the overall survival of patients with high-grade gliomas was significantly worse for high miR-9 expression group than for low miR-9 expression group ($p < 0.001$), but no significant difference was found for patients with low-grade (I–II) gliomas (Wu et al. 2013). Furthermore, miR-650 expression was increased in 168 gliomas (WHO I—35, WHO II—40, WHO III—41, WHO IV—52) compared with 21 normal control specimens ($p < 0.001$). It was also found that miR-650 expression was related to grade and KPS, whereas high expression was more frequently detected in gliomas of high grade or low KPS score ($p < 0.001$). The prognosis of glioma with high miR-650 expression was significantly worse compared with gliomas with low miR-650 expression (Sun et al. 2013).

MiR-21 is the most consistently overexpressed miRNA in many cancers including gliomas. To better understand the role of miR-21 in gliomas, paraffin-embedded glioma tissue samples from 193 patients with WHO I–IV tumors were analyzed by in situ hybridization. It was found that miR-21 expression is localized in tumor cells and tumor-associated blood vessels, whereas no expression was seen in adjacent normal brain parenchyma. Only tumor cell miR-21 was associated with poor prognosis when adjusting for known clinical parameters in a multivariate analysis (Hermansen et al. 2013). Similarly, miR-335 expression level was also described to be significantly higher than that in corresponding non-neoplastic brain tissues ($p < 0.001$) and, in addition, survival analysis demonstrated that patients with high miR-335 expression tumors had significantly shorter survival times ($p = 0.01$) and that miR-335 is an independent prognostic factor ($p = 0.02$) (Jiang et al. 2012). Similarly, miR-17, miR-224, and miR-372 are overexpressed in gliomas and their higher expression levels are significantly associated with worse progression-free survival and overall survival of glioma patients. Multivariate Cox regression analyses indicated that all these miRNAs are independent prognostic factors (Lu et al. 2012, 2013; Li et al. 2013a).

4.5 MicroRNAs Involved in Chemoradioresistance of Gliomas

DNA damage, caused by radiation, activates pathways that protect cells from apoptosis and give them the ability to grow and proliferate. There are two well-known pathways: PI3K/AKT and ATM (ataxia telangiectasia mutated)/Chk2 (checkpoint kinase 2)/p53 that are activated as a response to the treatment. These pathways are regulated by several miRNAs. As for radiation, DNA damage is the main reason for cell death in normal cells, where AKT is important activator of multiple proteins, such as DNA-PK (protein kinase, DNA-activated, catalytic polypeptide), which subsequently leads to DNA repair, BCL-2, or mTOR [mechanistic target of rapamycin (serine/threonine kinase)], which ensures cell growth and

proliferation. Next possibility of cells to recognize DSB (double strand breaks) or DNA damage is via BRCA1 (breast cancer 1, early onset) and activation of ATM/CHK2/p53 signaling pathway. The survival and cell apoptosis is dependent on activation of downstream proteins of this pathway.

Among treatment approaches that modulate and target DNA, TMZ is one of the currently frequently used. TMZ is an alkylating agent, which methylates nucleotides and so causes inhibition of DNA replication. Resistant cancer cells have evolved ways how to overcome this process. First mechanism is based on the MGMT enzyme that is able to excise nucleotides in DNA base-pair mismatch, and so neutralize the effect of TMZ. Other mechanism is efflux of TMZ by ATP-binding cassette transporters, which decrease the concentration of the drug within the cell. ATP-binding cassettes are probably the main reason for multidrug resistance. These processes were experimentally proved to be re-regulated by miRNAs (Fig. 4.6).

4.5.1 MicroRNAs Involved in Radioresistance

PI3K/AKT (Lee et al. 2011a) and ATM/CHK2/p53 (Squatrito et al. 2010) are two main pathways involved in radioresistance. These pathways are responsible for either activation of downstream proteins involved in repair of damaged DNA caused by radiation or regulation of apoptosis and survival rate (Li et al. 2009a). EGFR which triggers PI3K/AKT signaling pathways is important for proliferation activity of cells and is highly expressed in various types of cancer including gliomas. Furthermore, there is substantial experimental evidence supporting a causal role for aberrant EGFR signaling in cancer pathogenesis and resistance to treatment (Huang et al. 2009; Hatanpaa et al. 2010). PI3K is crucial for normal brain development and function. It was found to be hyperactivated in brain tumors (Guillamo et al. 2009; Kwiatkowska and Symons 2013). Studies concerning miRNAs and resistance revealed a connection between miRNAs and downstream proteins of EGFR/PI3K/AKT signaling.

PI3K/AKT signaling pathway is usually activated in GBM. Inactivation of this pathway impairs mechanisms of DNA repair, which can consequently enhance radiosensitivity. MiR-21 was shown to activate this pathway through suppression of PI3K/AKT inhibitor PTEN (Chakravarti et al. 2004; Kao et al. 2007; Gwak et al. 2012). MiR-21 belongs to one of the most studied miRNAs across many tumors including gliomas and is classified as an onco-miR. (Hong et al. 2013). It seems that miR-21 act as a major player in radioresistance, as decrease of this miRNA sensitizes GBM cell lines to irradiation. Sensitization of cells is mediated via γ -H2AX (H2A histone family, member X, gamma) blocking, which serves as an indicator of DSB and subsequently leads to prolongation of DNA repair (Gwak et al. 2012).

Further studies involved miR-7 in the regulation of PI3K/AKT signaling (Kefas et al. 2008). The involvement of miR-7 in this pathway was evaluated on two glioma cell lines U251 and U87, where increased level of miR-7 induced attenuation of EGFR and AKT, and caused a radio-sensitization (Lee et al. 2011a). AKT is

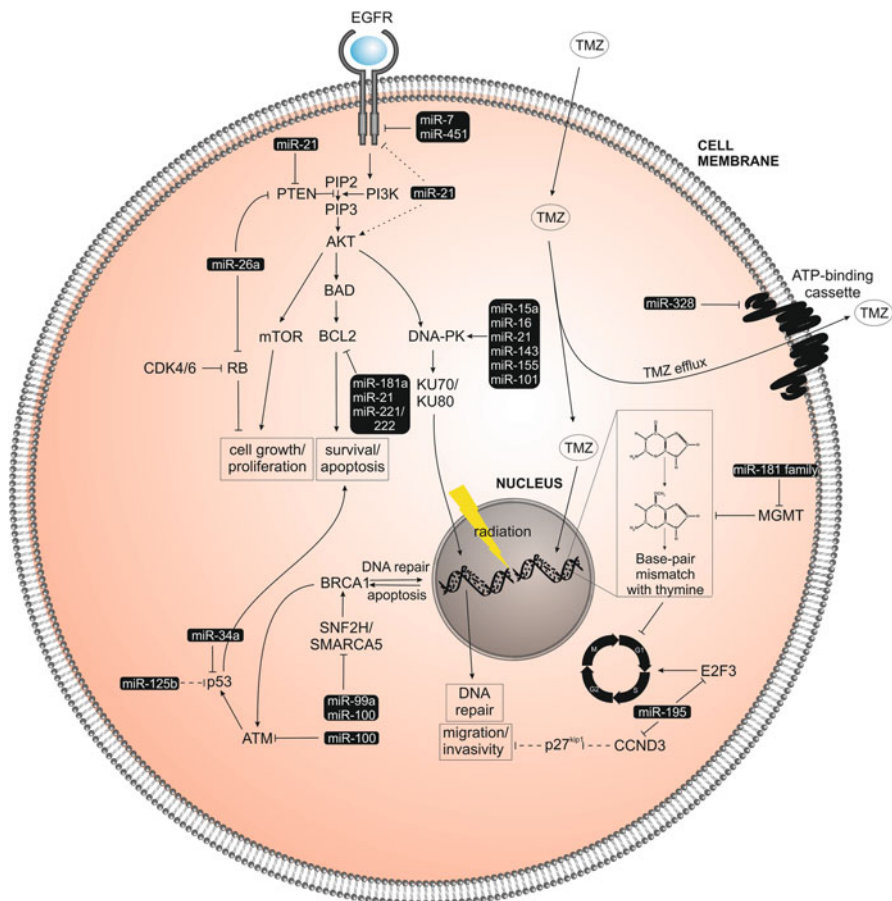


Fig. 4.6 MicroRNAs involved in radiotherapy and chemotherapy resistance. *AKT* v-akt murine thymoma viral oncogene homolog; *ATM* ataxia telangiectasia mutated; *BAD* BCL2-associated agonist of cell death; *BCL2* B-cell CLL/lymphoma 2; *BRCA1* breast cancer 1; *CCND3* cyclin D3; *CDK4/6* cyclin-dependent kinase 4/6; *DNA-PK* protein kinase, DNA-activated, catalytic polypeptide; *E2F3* E2F transcription factor 3; *EGFR* epidermal growth factor receptor; *KU70/80* X-ray repair complementing defective repair in Chinese hamster cells 5/6; *MGMT* O-6-methylguanine-DNA methyltransferase; *mTOR* mechanistic target of rapamycin (serine/threonine kinase); *p27* protein 27; *p53* protein 53; *PI3K* phosphatidylinositol-4,5-bisphosphate 3-kinase; *PIP2* phosphatidylinositol 4,5-bisphosphate; *PIP3* phosphatidylinositol (3,4,5)-triphosphate; *PTEN* phosphatase and tensin homolog; *RB* retinoblastoma; *SMARCA5* (*SNF2H*) SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 5; *TMZ* temozolomide. —| direct suppression; —> direct activation; ---| indirect suppression; ---> indirect activation

also important player in DNA damage repair as it controls expression of DNA-PK. DNA-PK serves as an activator of heterodimers Ku70/Ku80 (X-ray repair complementing defective repair in Chinese hamster cells 5/6), which are able to recognize DNA DSB during NHEJ (non-homologous end joining), and so it enables

to repair DNA after DNA damage (Fell and Schild-Poulter 2012). As by decrease of miR-21, also miR-7 was able to regulate response of cell to DNA damage, through regulation of DNA-PK. This indicates the relationship between delayed DNA damage repair and increased radiation-induced cell killing by miR-7 overexpression. It seems that miR-7 could be a useful therapeutic target for overcoming the radioresistance of GBM and other tumors with activated EGFR-PI3K-AKT signaling, as it had an effect in different cancer types treated with radiation (Lee et al. 2011a).

MiR-181a is next miRNA which has been described in connection with GBM and radiation treatment. It affects anti-apoptotic gene *BCL2* and it was shown that irradiation exhibited and induced expression of miR-181a, while overexpression of miR-181a caused sensitization to radiation (Chen et al. 2010a).

ATM/Chk2/p53 pathway is closely connected to cellular processes such as regulation of apoptosis, cell cycle, or senescence (Cao et al. 2006). The importance of ATM level in radioresistance was shown on two GBM cell lines, which differ in ATM expression. An M059J radiosensitive cell line is characterized by lower level of ATM and M059K radioresistant cell line, which show higher expression of ATM due to deficiency in DNA-PK. There is an evidence that miR-100 could be involved in this process, as it is differently expressed in these cell lines and accordingly to the computational analyses is a direct regulator of ATM (Ng et al. 2010). Irradiation of M059K and M059J cell lines caused upregulation of several miRNAs: miR-17-3p, miR-17-5p, miR-19a, miR-19b miR-142-3p, and miR142-5p. However, cell line with lower level of ATM and normal DNA-PK activity exhibited induction of miR-15a, miR-16, miR-21, miR-143, and miR-155 level (Chaudhry et al. 2010b). Establishment of these miRNAs in radiation response was confirmed by other studies. Study focused on lymphoblastoid cell revealed that low dosage of IR elevates expression level of miR-15a, miR-16, and miR-21 (Chaudhry et al. 2010a). Mir-143 was found to directly target gene *FHIT* (fragile histidine triad) the deregulation of which is often seen in epithelial tumors and homozygous deletion contributes to higher radioresistance. Regulation of FHIT was shown via miR-143 overexpression which consequently leads to cell cycle arrest in G2 phase (Lin et al. 2011). It was also shown that elevated level of miR-155 serves as a radioprotectant in lung cancer cells. Inhibition of miR-155 led to radio-sensitization (Babar et al. 2011). ATM and DNA-PK were confirmed as targets of miR-101. These miRNAs sensitized U87MG glioma cells to radiation after lentiviral transduction in vitro and in vivo (Yan et al. 2010).

For radioresistance of cells it is very important to recognize and repair DSB or DNA damage, chromatin remodeling protein complex. A positive correlation between miR-99 and SWI/SNF (SWI/SNF/Sucrose NonFermentable) chromatin remodeling factor SNF2H/SMARCA5 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 5), a component of the ACF1 (bromodomain adjacent to zinc finger domain, 1A) complex, was found. Moreover, it has been elucidated that reduction of BRCA1 level on DNA damage site was arranged due to the downregulation of SNF2H, miR-99a, and miR-100. Ectopic expression of the miR-99 family in cells reduced the rate and overall

Table 4.3 Effects of microRNAs involved in regulation of radioresistance in gliomas

miRNA	Up/ downregulation	Effect on radioresistance	Targets	References
miR-181a	↓	+	BCL-2	Chen et al. (2010a)
miR-7	↓	+	EGFR, AKT, DNA-PKs	Lee et al. (2011b)
miR-101	↓	+	ATM, DNA-PK	Yan et al. (2010)
miR-99	↑	–	SNF2H, SMARCA5	Mueller et al. (2013)
miR-100	↑	–	SNF2H, SMARCA5	Ng et al. (2010)
miR-21	↑	–	γ-H2AX, AKT	Gwak et al. (2012), Hong et al. (2013)
miR-143	↑	+	FHIT	Lin et al. (2011)
miR-155	↑	+	FOXO3a	Ling et al. (2013)
miR17-3p	Induction after IR		MDM2	Chaudhry et al. (2010b), Wang et al. (2012a)
miR17-5p	Induction after IR		PTEN	Chaudhry et al. (2010b), Wang et al. (2012a)
miR-19a	Induction after IR			Chaudhry et al. (2010b), Jia et al. (2013)
miR-19b	Induction after IR			Jia et al. (2013)
miR142-3p	Induction after IR			Chaudhry et al. (2010b)
miR142-5p	Induction after IR			Chaudhry et al. (2010b)

Adapted from Besse et al. (2013)

efficiency of repair by both homologous recombination and non-homologous end joining, which led to sensitization of cells to radiation (Nakano and Kornblum 2006; Mueller et al. 2013; Besse et al. 2013). Effects of miRNAs and their targets are summarized in Table 4.3 and Fig. 4.6.

4.5.2 MiRNAs Involved in Chemoresistance

Temozolomide is a cytotoxic prodrug agent, which after hydrolyzation methylates nucleotides and causes inhibition of DNA replication. The resistance after long-time exposure is nearly universal. This is caused by three main mechanisms involved in DNA repair: increased levels of MGMT; a deficient mismatch repair process (MMR); and activation of the PARP (poly(ADP)-ribose polymerase) pathway (Darkes et al. 2002; Carmo et al. 2011).

MGMT repairs mutagenic DNA lesions, caused by TMZ guanines methylation on O⁶ position that subsequently leads to base-pair mismatch with thymine. Further,

MGMT prevents mismatches and errors during DNA replication and transcription. Cells with increased level of MGMT become resistant to the exposure of TMZ, while in nonresistant glioma cells the pathway induced by TMZ is active and is able to induce cell death (Darkes et al. 2002; Sharma et al. 2009).

As described previously, miRNAs have the possibility to regulate multiple genes, as was observed in miR-181, which is involved in the regulation of BCL2 and MGMT. Silencing of miR-181 family led to increased level of MGMT. Therefore, miR-181 could be used as a predictive factor to TMZ response (Fig. 4.6) (Chen et al. 2010a; Zhang et al. 2012c).

Correlation of TMZ chemoresistance and survival pathways PI3K/AKT and ERK1/2/MAPK (mitogen-activated protein kinase) was confirmed on U118 cell line, which evinces chemoresistance, while after inhibition of these pathways chemoresistance was partially eradicated (Carmo et al. 2011).

Regulation of PI3K/AKT by miR-21 was described also by TMZ-treated glioma cell lines. Decreased level of this miRNAs caused inhibition of proliferation, increased apoptosis, and cell cycle arrest. This was particularly caused by the decrease of BAX (BCL2-associated X protein)/BCL-2 ratio and decreased caspase-3 activity (Ren et al. 2010b; Shi et al. 2010). Among miRNAs targeting apoptotic factors such as BAX, cytochrome c, and cleaved caspase-3 belongs miR-221/222 as well. Their decreased levels enhanced sensitivity to TMZ, but independently of the p53 status (Chen et al. 2012).

To evaluate differences in cell resistant to TMZ, two cell lines U251R, U251 were compared on their miRNA profile. This revealed an elevated level of miR-195, miR-455-3p, and miR-10a* in U251R (TMZ-resistant) cells, which could be important in TMZ resistance. Inhibition of miR-455-3p or miR-10a(*) showed modest cell killing effect in the presence of TMZ. However, miR-195 with TMZ in combination strongly increased cell death (Ujifuku et al. 2010). This was caused by inverse correlation of E2F3 (E2F transcription factor 3) and CCND3 (cyclin D3) and expression level of miR-195. These results indicate that E2F3 and CCND3 are targets of this miRNA. E2F3 is able to suppress transcription of genes related to cell cycle progression and so causes G1 phase arrest. On the other hand CCND3 suppression mediated by miR-195 allows an increase of p27Kip1 (cyclin-dependent kinase inhibitor 1B) expression level, which consequently influences proteins involved in cell migration, resulting in repression of GBM cell invasion (Zhang et al. 2012a).

Although TMZ is the most often used chemotherapeutical agent, there are other chemotherapeutics used in GBM treatment. VM-26 (Teniposide) was tested on U373MG cell line in combination with miR-21 inhibitor, which led to enhancement of cytotoxicity. As a direct target LRRFIP1 (leucine-rich repeat (in FLII) interacting protein 1) was validated, whose product is an inhibitor of NF- κ B, a downstream effector of PI3K/AKT signaling (Li et al. 2009b). MiR-21 effect was also examined in cells treated by 5-FU (5-fluorouracil) and showed increased apoptosis and decreased ability to migrate (Ren et al. 2010a). Not only deregulation of PI3K pathway and its downstream factor is responsible for resistance, but also family of proteins called *ABCG2* (ATP-binding cassettes, a subfamily of G member

Table 4.4 Effects of miRNA involved in regulation of chemoresistance in gliomas

miRNA	Up/ downregulation	Effect on chemoresistance	Targets	References
miR-181	↓	–	MGMT	Zhang et al. (2012c)
miR-21	↑	–	BAX, BCL-2	Shi et al. (2010)
miR-221/ 222	↓	–	BAX, cyt c, Caspase3	Li et al. (2009b)
miR-195	↑	+	ENF3, CCND3	Chen et al. (2012)
miR-455- 3p	↑	+		Zhang et al. (2012a)
miR-10a*	↑	+	EPHX1, BED7	Ujifuku et al. (2010)
miR-328	↑	–	ABCG2	Li et al. (2010)

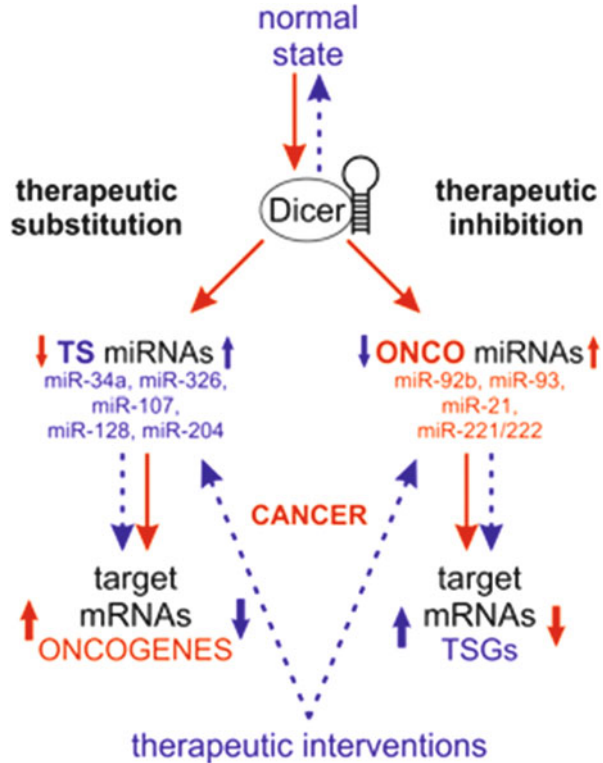
Adapted from Besse et al. (2013)

2 protein family), which contribute to multidrug resistance. These transporters cause selective survival of cells through the efflux of drugs. MiR-328 contributes to the regulation of *ABCG2* expression, while increased level of this miRNA led to decrease of *ABCG2* expression (Li et al. 2010). Regulatory effects of miRNAs involved in chemoresistance of gliomas are summarized in Table 4.4 and Fig. 4.6.

4.5.3 Glioma Initiating Cells/Stem Cells and Their Role in Tumor Resistance

GSCs, which maintain stem-like phenotype, show generally much more resistance to radiation or chemotherapy in comparison to “normal” glioma cells (Wang et al. 2010; Yamada and Nakano 2012). Therefore, it seems that the best way how to decrease the number of recurrences which is connected with high treatment resistance is to target these cells and their signaling pathways. This population of CD133 positive (CD133+) cancer stem cells contributes to the radio- and chemoresistance and bears the activation of NOTCH and SHH (Sonic hedgehog) pathways. Inhibition of these pathways led to enhancement of CD133+ glioma cells to TMZ treatment (Ulasov et al. 2011). Further, SHH has been found to play a role in maintaining undifferentiated stem cell state in GSCs and Notch signaling in active GSCs as well as in GBM pathogenesis (Yamada and Nakano 2012). In fact, NOTCH and SHH were shown to be upregulated in GSCs and their inhibition led to increased susceptibility of these cells to TMZ. Involvement of miRNAs in regulation of stem-like phenotype of GSCs is described in detail in Sect. 4.3.6.

Fig. 4.7 MicroRNAs as a potential therapeutic target in gliomas; see text



4.6 MicroRNA as Therapeutic Targets

The role of miRNA in carcinogenesis depends on the functions of their targets (oncogenes or tumor suppressors). Downregulated miRNAs, which regulate oncogenes, are tumor-suppressive miRNAs (TS-miRNAs), while miRNAs overexpressed in tumors and inhibiting tumor suppressors are classified as an onco-miRs. Therefore, there are two possible miRNA-based strategies: therapeutic inhibition (in case of onco-miRs) or substitution (in case of TS-miRNAs) (Fig. 4.7).

Specific inhibitory effect of miRNAs can be achieved by use of their complementary antagonists (anti-miRs). Whereas in the most cancers tumor tissues indicate global decrease in miRNA expression levels, therapeutic substitution presents the most promising approach. So far, many studies have described oncogenic/tumor-suppressive miRNAs and their contribution to development and progression of gliomas. Most of these studies were performed under *in vitro* conditions (Li et al. 2013b). *In vitro* models are suitable for studies on specific targets and regulatory pathways; however, to evaluate the real therapeutic potential, it is necessary to study miRNAs on animal models. MiRNAs *in vivo* studies are mainly

focused on the evaluation of particular miRNA effects on the tumor growth and confirmation of phenotypic observations from in vitro studies.

It is presumed that GSCs may stand for the cells responsible for tumor formation and treatment failure. Notch signaling is important for maintaining this stem-like phenotype and has been found to be aberrantly expressed in GSCs. Inhibition of this signaling pathway decreases proliferation, increases neuronal differentiation, and reduces CD133+ cell fraction in vitro; moreover, in vivo such inhibition attenuates tumorigenicity (Stockhausen et al. 2010). MiR-34a is often described as direct regulator of Notch signaling and could serve as potential tumor suppressor in brain tumors. This was demonstrated by transfection of pre-miR-34a to cells, which were consequently implanted to immunocompromised mice. Four weeks after implantation, xenografts of pre-miR-34a-transfected cells developed tumor of significantly smaller volume compared to control cells (Li et al. 2009a, 2011). Moreover, other miRNAs were confirmed as direct regulators of Notch signaling, such as miR-326 and miR-107. Similarly to miR-34a, exogenous expression of these miRNAs in glioma cells caused decrease of the tumor bearing in mice (Kefas et al. 2009; Chen et al. 2013a). Inhibition of NOTCH can be also achieved by miR-92b. It was shown that this miRNA regulates NLK (Nemo-like kinase), which negatively regulates NOTCH-dependent transcriptional activation. Furthermore, expression of NLK was inversely correlated with miR-92b in clinical glioma samples and enables prediction of clinical outcome. An effect of miR-92b on glioma tumors was evaluated in vivo by using nude mice. Glioma cell lines were subcutaneously injected to mice and after reaching 100 mm³ tumor volumes were animals treated with anti-miR-92b oligonucleotide. Anti-miR-92b triggered growth inhibition, induced apoptosis, and suppressed invasion of glioma (Ishitani et al. 2010; Wang et al. 2013a).

Consistently, miR-204 was found to be downregulated in glioma cells and also in neural stem cells. Inhibition of miR-204 promoted not only cellular stemness, but also the biological properties essential to lethality of the disease; however, restoration of miR-204 greatly abrogates the aggressiveness of glioma cells. This observation is supported by Kaplan–Meier survival analysis of mice transduced by miR-204 compared to control. MiR-204 prolonged the survival of mice by approx. 15 %. This is probably caused by miR-204 targeting of *SOX4* gene, which is the core regulator governing the stemness of both glioma and neural stem cells (Ying et al. 2013).

Aggressiveness of stem-like cells and ability to form new in situ tumors is closely related to proliferation and invasive ability. Receptor tyrosine kinases such as EGFR and PDGFR play a major role in glioma proliferation and invasion (Nakada et al. 2013). In gliomas, regulation of these receptors by miRNAs was observed in many in vitro studies, which were consequently confirmed in animal models. It was shown that miR-128 represses GSC growth by enhancing neuronal differentiation and mediates differentiation by targeting EGFR and PDGFR α . Authors of this study proved this by utilization and autochthonous glioma mouse model, which arises from activation of oncogenic H-RasV12 (Harvey-RasV12) and loss of p53. Developed glioma tumors in mice showed significant decrease of

miR-128 and high levels of PDGFR α compared to normal brain tissue. The introduction of miR-128 led to remarkable improvement of survival in treated animals. This study validated miR-128 as effective suppressor of glioma growth (Papagiannakopoulos et al. 2012).

Another interesting example of onco-miR is miR-93. It enhances tumor cell survival, blood vessel expansion, and tumor growth, by targeting, at least in part, integrin- β 8 expression. Consistent with this was the decreased survival rate of mice bearing the miR-93 positive tumors compared with the controls and Kaplan–Maier survival curves which indicated that miR-93 expression decreased rates of mouse survival. Further, higher levels of integrin- β 8 are associated with cell death in tumor mass and in human glioblastoma (Fang et al. 2011). Accelerated proliferation is a characteristic for tumor cells and contributes to excessive growth and tumor progression. *PDCD4* is a well-known tumor-suppressor gene. Overexpression of *PDCD4* caused by downregulation of miR-21 led to decreased proliferation and apoptotic rate and in the xenograft model decreased tumor formation and growth (Gaur et al. 2011b). Clinical relevance of miR-21 was further studied by Xuan Zhou et al. In the in vivo pharmacological study 30 mice were included and screened for tumor volume of xenografts treated with PBS, negative control, and anti-sense-miR-21 (as-miR-21). Significant decrease in tumor volume was observed only in as-miR-21-treated group. These data suggest miR-21 as a promising therapeutic target for malignant gliomas (Zhou et al. 2010a).

Even if miRNA-based therapies were not clinically implemented to the therapy, they have several important advantages to conventional drugs and targeted therapy in oncology. MiRNAs have ability to target multiple genes, which give them a priority in cancer treatment, considering cancer as a heterogeneous disease with multiple molecular alterations. In addition, miRNAs are not detected by immune system and so they could be an attractive option for clinical drug development.

Conclusions and Future Perspectives

The discovery of miRNA function has markedly spread the view on regulation of gene expression. Its remarkable ability to regulate large number of genes, including oncogenes and tumor-suppressor genes, has catapulted miRNAs into the center of cancer molecular biology over the past 5 years. It is now evident that dysregulation of miRNAs is an important step in the development of many cancers, including gliomas. Several studies based on expression profiling have proved that there are significant changes of miRNA expression levels in gliomas compared to non-tumor brain tissue. These studies also identified groups of miRNAs with potential for prognostic stratification and prediction of responses to chemoradiotherapy in glioma patients. But much more studies have been focused on the improvement of our knowledge about miRNAs involvement in glioma core signaling pathways. The results of these studies suggest a great potential and relevance of miRNAs as a novel class of therapeutic targets and possibly powerful intervention tools in glioma.

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